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The 17-Gene Ethanolamine (*eut*) Operon of *Salmonella typhimurium* Encodes Five Homologues of Carboxysome Shell Proteins

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The *eut* operon of *Salmonella typhimurium* encodes proteins involved in the cobalamin-dependent degradation of ethanolamine. Previous genetic analysis revealed six *eut* genes that are needed for aerobic use of ethanolamine; one (*eutR*), encodes a positive regulator which mediates induction of the operon by vitamin B_{12} plus ethanolamine. The DNA sequence of the *eut* operon included 17 genes, suggesting a more complex pathway than that revealed genetically. We have correlated an open reading frame in the sequence with each of the previously identified genes. Nonpolar insertion and deletion mutations made with the Tn*10*-derived transposable element T-POP showed that at least 10 of the 11 previously undetected *eut* genes have no Eut phenotype under the conditions tested. Of the dispensable *eut* genes, five encode apparent homologues of proteins that serve (in other organisms) as shell proteins of the carboxysome. This bacterial organelle, found in photosynthetic and sulfur-oxidizing bacteria, may contribute to CO_2 fixation by concentrating CO_2 and excluding oxygen. The presence of these homologues in the *eut* operon of *Salmonella* suggests that CO_2 fixation may be a feature of ethanolamine catabolism in *Salmonella*.

Under aerobic conditions, *Salmonella typhimurium* can use ethanolamine as a sole source of carbon, nitrogen, and energy (44, 47). However, this growth depends on exogenous cobalamin, a required cofactor that *Salmonella* cannot synthesize in the presence of oxygen. Under anaerobic conditions, vitamin B_{12} is made, but *Salmonella* cannot use ethanolamine as a carbon or energy source, even with the alternative electron acceptor nitrate or fumarate. Recently this paradox has been resolved by the finding that the anaerobic electron acceptor tetrathionate allows *Salmonella* to use endogenous B_{12} to support anaerobic degradation of ethanolamine as a sole source of nitrogen, carbon, and energy (12). Anaerobic use of ethanolamine may be important to *Salmonella*, since this carbon source is a constituent of an abundant class of lipids which would be provided to anaerobic gut inhabitants as part of the host's dietary intake.

The initial genetic analysis of the *eut* operon was done with mutants defective in aerobic degradation of ethanolamine on medium including cobalamin. A large set of mutations were sorted into six complementation groups (*eutABCDER*) and ordered by deletion mapping (44, 45). More recent genetic tests have identified a seventh complementation group, *eutT* (54, 67).

The standard reactions in ethanolamine utilization are diagrammed in Fig. 1, with proposed roles for several Eut proteins. One previously identified gene, *eutR*, encodes a positive regulatory protein which mediates induction of the operon by ethanolamine plus cobalamin (46, 53). Two genes (*eutBC*) encode subunits of the cobalamin-dependent ethanolamine ammonia lyase (27, 45), which converts ethanolamine to acetaldehyde and ammonia (13, 50). The *eutE* gene encodes the second enzyme in the pathway, acetaldehyde dehydrogenase, which forms acetyl-coenzyme A (CoA) (45). As expected, bacteria with mutations in this gene can use ethanolamine as a source of nitrogen but not carbon [a Eut(N^+ C^-) phenotype]. The EutT enzyme appears to be an adenosyl transferase, converting CNB_{12} to $AdoB_{12}$, and the EutA protein appears to protect the lyase (EutBC) from inhibition by CNB_{12} (54). We propose below that the *eutG* gene encodes an alcohol dehydrogenase. No function has been assigned to the *eutD* gene, whose mutants have a Eut(N^+ C^-) phenotype.

We report here the complete DNA sequence of the *eut* operon and adjacent regions, including about 7 kb of new sequence and several corrections of previously reported data. Previously sequenced parts of the operon include the *eutB* and *eutC* genes (27) and a nonoverlapping 8-kb fragment (60). Surprisingly, the operon includes 17 open reading frames, suggesting that 11 *eut* genes escaped detection by the initial genetic analysis. Here we correlate the genetic and physical maps of the operon and analyze available information on the function of each of the 17 genes. Using insertions of a new transposon (T-POP) and derived deletion mutations, we provide evidence that at least 10 of the 11 extra genes are not needed for aerobic ethanolamine metabolism. Five of the extra *eut* genes encode homologues of three families of proteins that serve in other prokaryotes as shell proteins of the carboxysome, an organelle which stimulates CO_2 fixation and has been suggested to concentrate CO_2 (3, 25, 29, 57). We propose that a similar organelle forms in *Salmonella* and supports catabolism of ethanolamine by a route that involves CO_2 fixation.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derivatives of *S. typhimurium*; in view of the large number of strains used, strain numbers are listed only in data tables and in the text. Isolation of all *zfa* insertions (near the *eut* operon) and all *eut* mutations with allele numbers below 205 was described previously (44–46). Transposon Tn*10*dTc is a transposition-defective derivative of transposon Tn*10* (68). The T-POP transposon, derived from Tn*10*dTc, directs tetracycline-inducible promoters into genes adjacent to its insertion site (42).

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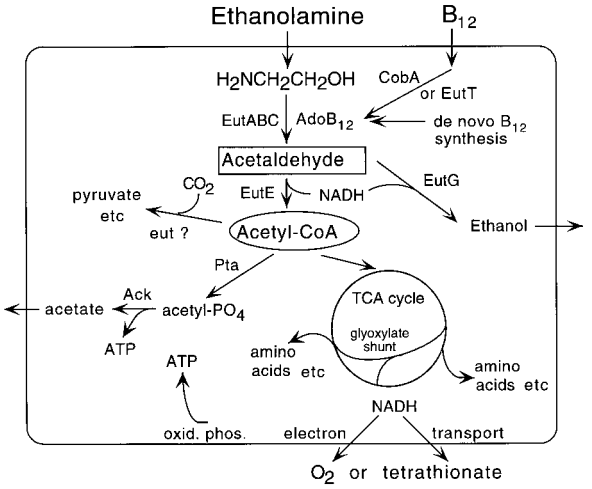

FIG. 1. Suggested pathway for metabolism of ethanolamine. Gene assignments are based on direct assays (EutBCE and CobA), on mutant phenotypes (EutA, EutT, Ack, Pta, and glyoxylate shunt), or on sequence similarity (EutG). The homologues of carboxysome shell proteins suggest the possibility of CO_2 fixation, which has not been demonstrated. In the diagram, the outer boundary is the cell membrane; the role of carboxysomes in this pathway is unknown.

MudA and MudJ elements are transposition-defective derivatives of phage Mu (15, 16). MudP and MudQ (MudP22 elements) have the ends of phage Mu but include a chloramphenicol resistance determinant and a P22 prophage that cannot excise when induced but packages a limited region of the chromosome adjacent to the MudP or -Q insertion site (70). Strains carrying MudP or MudQ insertions near the *eut* operon were induced in order to obtain template DNA for sequencing the *eut* operon.

Media, chemicals, and enzymes. The rich medium was Luria-Bertani broth. The carbon-free minimal medium was NCE (5), and the carbon- and nitrogen-free minimal medium was NCN (43). Ethanolamine hydrochloride (3) at 0.4% was used as a carbon source in the last two media. MacConkey agar base (Difco) was used as a colorimetric indicator of acid production and was prepared according to the manufacturer's specifications.

When used, antibiotics were present at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 20 μg/ml (selection) or 2 μg/ml (T-POP transcription); chlortetracycline, 10 μg/ml (T-POP transcription); kanamycin, 50 μg/ml; and chloramphenicol, 20 μg/ml. Chlortetracycline was activated by autoclaving it with the medium. The chromogenic β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Diagnostic Chemicals) was used at a final concentration of 0.01%. Cyanocobalamin (CN-B_{12}; Sigma) was used at a final concentration of 200 nM.

Crystalline bovine serum, Ficoll (type 400), and cresol red were from Sigma. Premixed deoxynucleoside triphosphates were from Pharmacia. Isotopically labelled nucleotides (^{32}P and ^{33}P) were from Dupont, New England Nuclear. Hexadecyltrimethylammonium bromide (CTAB) was from Aldrich. *Taq* polymerase was purchased from Promega, TaqStart antibody was from Clontech, and proteinase K was from Gibco-BRL.

Genetic techniques. Transduction crosses were mediated by the high-frequency generalized transducing phage P22 HT105/1 *int*-201 (51). Transductants were freed of phage by streaking them on green indicator plates (17). Cells were cross-streaked with the P22 clear plaque mutant H5 to verify phage sensitivity.

Selecting insertions of T-POP in the *eut* operon. The T-POP derivative of transposon Tn*10* directs tetracycline-induced promoters out of each end (42). In this cross, the donor (TT18797) carried the T-POP insertion on an *Escherichia coli* F′, plasmid; the lack of homology prevents recombination between the transduced T-POP region and the recipient chromosome. For some crosses, the recipient (TT17428) carried a standard Tn*10* transposase (plasmid pZT380); in other cases, the recipient (TT17437) expressed a mutant form of IS*10* transposase (plasmid pNK2881) that allows transposition with relaxed target site specificity (4). Selected tetracycline-resistant clones inherited T-POP by transposition into random sites in the chromosome. A large collection (>10,000) of random-insertion clones were pooled to create the T-POP pool.

Transducing phage prepared on the T-POP pool were used to transduce a recipient that carried a *eutR*::MudJ insertion; the *lacZ* gene of this recipient is not expressed, since it lacks the EutR protein required for operon induction. Clones were sought which formed red (Lac^+) colonies on MacConkey agar-lactose-tetracycline plates (due to the T-POP promoter) and white colonies without tetracycline (when the T-POP promoter is repressed).

Making deletion mutations by using insertions of T-POP. Phenotypically Eut^- insertion mutants were subjected to selection for aerobic growth on ethanolamine plus vitamin B_{12}. Some surviving clones carried a deletion that removed the inserted material and extended into adjacent regions of the *eut* operon that are not essential to the Eut^+ phenotype. Four different in-frame Eut^+ deletions that lie between the promoter and the *eutR* gene were isolated; each was made from a different parental *eut* insertion.

Preparation of chromosomal DNA. Crude template DNA for rapid PCR mapping was prepared by resuspending a 50-μl cell pellet of an overnight culture in Tris-EDTA (TE) buffer, holding it at 95°C for 3 min, spinning out cell debris, and using the supernatant directly. These preparations lost template efficiency with repeated freezing and thawing or storage on ice and were unsatisfactory for sequencing.

Chromosomal DNA preparations for sequencing were prepared as suggested by Knut Jahreis (personal communication). A fresh overnight cell culture (1.5 ml) was centrifuged and resuspended in 567 μl of TE buffer (10 mM Tris, pH 8.3, 1 mM EDTA). Sodium dodecylsulfate (15 μl of a 20% solution) and proteinase K (3 μl of a 20-mg/ml solution) were added, and the suspension was incubated for 1 h at 37°C. NaCl (100 μl of a 5 M solution) was then added with gentle but complete mixing. CTAB was then added (80 μl of a solution of 41 mg of NaCl and 100 mg of CTAB in 1 ml of H_2O) with gentle mixing. After 10 min at 65°C, the mixture was extracted with 1 volume of CIA (chloroform-isoamyl alcohol [24:1]). The aqueous phase was saved and drawn repeatedly through a 22-gauge syringe needle to fragment the DNA. The preparation was then extracted twice with phenol-CIA (1:1), and the final aqueous phase was extracted with 1 volume of 1-butanol. DNA was precipitated by addition of 1 volume of isopropanol and was recovered by centrifugation. The pellet was washed once with 70% ethanol, placed under vacuum until nearly (but not completely) dry, resuspended in 100 μl of H_2O, and stored at −20°C.

PCR methods. (i) Standard amplification techniques. All PCRs were done in glass capillaries with an AirCycler thermal cycler (Idaho Technology). The buffers and conditions were as described in protocols provided by the company, with the following modifications: cresol red was used as the indicator dye, and magnesium was used at 1, 2, or 3 mM. Products for sequencing were purified with Wizard PCR purification kits (Promega). The two methods described below were used to amplify unknown sequence adjacent to a single known region.

(ii) Semirandom amplification. At sufficiently low stringency, a primer will often misprime close enough to its correct binding site that amplification of the intervening DNA will occur with a single primer (P1). The most stringent conditions of magnesium and annealing temperature which still allow one or a small number of misprimed bands to form are determined. These bands are excised and used as templates in a reamplification reaction at high stringency, using P1 with a nested primer oriented in the same direction (P2) at a 1:100 molar ratio of P1 to P2. P1 will continue to initiate at the unknown end, but P2 will dominate priming at the known end, leading to amplification of a fragment differing in size from the original product by the distance between the ends of P1 and P2. This difference is diagnostic of a product derived from the known region. The technique generates template which can be sequenced from either end by using P1 or P2.

(iii) Nested amplification. The second method uses one primer in known sequence (K primer) and an ambiguous N primer, which is designed to misprime at nearby sites. The amplified region is between the known K primer and all of the sites at which the N primer acts. This method is sensitive to initial template complexity. It works well on DNA extracted from MudP22 heads or on large PCR products.

The four N primers had the sequence ACTTCTCAACAACTCAGGACGA $ACA(N)_{10}XCAGC$, where X is replaced by G, A, T, or C, yielding primers NG, NA, NT, and NC. The reamplification primer (P primer) is identical in sequence to the common portion of the initial oligonucleotide preceding the run of 10 ambiguous bases in the N primers.

Four initial primer extensions were done with NX primers at extremely low stringency (annealing temperature of 40°C). Wizard PCR columns were used to remove the primers and most of the large template DNA, which binds irreversibly to the columns. Extension times were less than 1 min, so most products are short enough to be easily eluted.

The extensions were then used as templates in standard amplification reactions containing a known primer and the shorter P primer, which recognizes the outside end of all NX-primed products. Reamplification with a nested known primer can be used to identify correctly anchored fragments. Optimally, amplification with a nested known primer and the P primer yielded a series of products separated by an average of 256 bases. The unknown ends of all products can be sequenced with the P primer.

Sequencing the *eut* operon and identifying insertion sites. Sequencing templates were PCR-amplified genomic regions between genetically mapped Tn*10* and Mud insertions or between one such insertion and previously determined *eut* sequence. The approximate positions of insertions were judged by the size of the fragment; the precise position was determined by sequencing the junctions between the element and adjacent chromosomal sequence. To amplify regions resistant to PCR, MudP22-packaged DNA was sequenced directly; this DNA was obtained from phage particles released after inducing one of the MudP or MudQ lysogens (TT14884, TT15254, or TT15632). MudP and MudQ elements are described above.

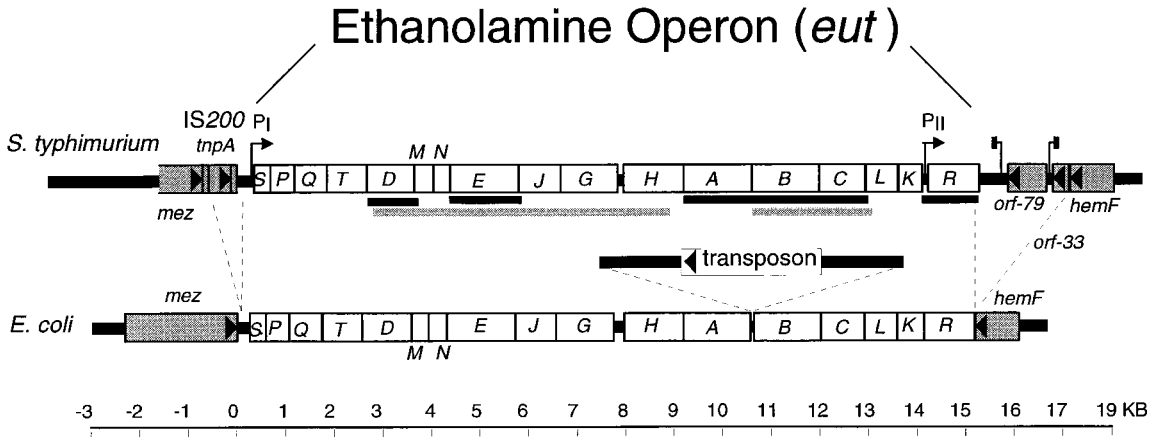

FIG. 2. Diagram of the *eut* operon sequence. The genes underlined in black were discovered by genetic characterization of mutants defective for aerobic use of ethanolamine. The regions underlined in gray were sequenced previously (27, 60). The transposon shown in the *E. coli* sequence was found in one isolate of *E. coli* (8) but not in another (69).

Sequencing was done according to the method of Sanger et al (49) with variations described in manganese reagent Sequenase or ThermoSequenase kits (Amersham Life Science) and in protocols for dye-terminator sequencing (Applied Biosystems). The latter was carried out at the University of Utah Health Sciences DNA Sequencing Facility, headed by Margaret Robertson. Primers were synthesized by Robert Schackmann at the University of Utah Health Sciences DNA/Peptide Synthesis Facility.

Nucleotide sequence accession number. The sequence described here has GenBank accession no. AF093749.

RESULTS

Catabolism of ethanolamine. A current view of ethanolamine catabolism is diagrammed in Fig. 1. This scheme is consistent with previous genetic analyses and includes some of the gene assignments proposed here. Acetyl-CoA is formed by the sequential activity of the vitamin B_{12}-dependent lyase and the dehydrogenase whose genes were identified genetically (*eutBC* and *eutE*). Acetyl-CoA can be converted to acetyl phosphate and excreted as acetate, yielding one molecule of ATP; these reactions (Pta and Ack functions) are required for aerobic growth on ethanolamine (28). Acetyl-CoA can enter the tricarboxylic acid (TCA) cycle and provide both a carbon and an energy source by respiration of oxygen. Under anaerobic conditions, tetrathionate can be used as an alternative electron acceptor, but other alternative acceptors, including nitrate and fumarate, do not support anaerobic growth on ethanolamine (12). The TCA cycle is thought to be essential, since mutants blocked in the glyoxalate shunt fail to use ethanolamine (12). If NADH generated by the TCA cycle exceeds that which can be removed by respiration, acetaldehyde may serve as an electron sink by being reduced to ethanol (Fig. 1). The energy yield from ethanolamine by these pathways might be expected to exceed that provided by acetate, because ethanolamine can enter cells by diffusion and be converted to acetyl-CoA by a dehydratase with no energetic cost. In contrast, acetate must be transported and converted to acetyl-CoA at the cost of at least one ATP.

Since the vitamin B_{12} cofactor of ethanolamine ammonia lyase is only made anaerobically, we suspect that under natural conditions a major use of ethanolamine may occur in the absence of oxygen. In the absence of any electron acceptor, conversion of ethanolamine to excreted acetate, catalyzed by the Pta and Ack activities, provides a source of energy (ATP) but not of carbon; this use of ethanolamine is detected as a stimulation of anaerobic growth on dilute casamino acids (12). When tetrathionate is provided as the alternative electron acceptor, ethanolamine can serve anaerobically as a nitrogen, carbon, and energy source, using endogenous vitamin B_{12}. Anaerobic growth on ethanolamine (or propanediol) with tetrathionate as an electron acceptor are the only conditions known to us under which vitamin B_{12} synthesis is required for growth of wild-type cells. We propose that many of the extra Eut enzymes may be involved in CO_2 fixation. This fixation may be required because so much carbon is lost as excreted acetate and ethanol (Fig. 1).

Sequence of the *eut* operon. The *eut* operon sequence was completed and is diagrammed in Fig. 2. The portions determined previously are indicated (27, 60). The operon includes 17 genes. This was surprising, because only six genes were identified genetically (*eutD*, *-E*, *-A*, *-B*, *-C*, and *-R*). Features of the sequence are listed in Table 1, and selected alignments with other genes are given in Table 2. A copy of the transposable element IS*200* was found upstream of the *eut* operon. Nucleotides are numbered with respect to the first base to the right of this IS*200* copy (Fig. 2). Sequence downstream of *eut* structural genes includes a probable transcription terminator and the nearby *hemF* gene. The sequence of the homologous region from *E. coli* is indicated for comparison and will be described later.

By investigating the function of each of the 11 extra genes, we hoped to gain a better understanding of ethanolamine metabolism. A series of *eut* mutations were characterized to demonstrate the mutant phenotype of each gene and to correlate the genetic and physical maps of the region. The mutant phenotypes explain why so many genes were missed in the initial genetic analysis of aerobic Eut^- mutants.

Correlating the genetic and physical maps of the *eut* operon. The physical locations of many genetically mapped insertions, deletions, and point mutations were determined from the sizes of PCR fragments or by sequencing. Table 3 lists the positions of insertion mutations. Correlation of these sites with the sites of genetically mapped deletion endpoints validates the genetic map (44) and supports the gene assignments listed below.

Deletion mutations (Table 4) were made from insertion mutations in two ways. Four deletion mutants (*eutPQTD*, *eutDM*, *eutJG*, and *eutGH*) were selected, each as a spontaneous Eut^+ derivative of a different *eut*::T-POP insertion; all four deletions are in frame and should not cause a polar effect on expression of downstream genes. Additional deletions were

TABLE 1. Features of the *eut* locus sequence

Name[a]	Gene organization[b]				Codons[c]				Protein			Comment
	Start	Stop	Sense	Overlap	GC	3d.GC	CAI	χ^2	Length (aa)	MW	pI	
'maeB	−1221	−766	→	−	0.55	0.60	0.292	0.817	>151	>1,610	6.9	One of two malic enzymes
IS*200*	−721	0	→									IS*200* element; copy V
tnpA	−578	−120	→	−	0.46	0.56	0.250	0.572	152	17,958	10.1	IS*200* transposase (Tnp)
orf'	1	57	←	−	0.58	0.79	0.192	1.926	19	1,907	7.5	*atsB*-like fragment
eutS	361	696	→	−	0.51	0.54	0.291	1.128	111	11,673	5.7	Carboxysome structural protein?
eutP	709	1188	→	+	0.52	0.54	0.243	0.645	159	17,726	5.9	Unknown function; ATP binding motif
eutQ	1166	1855	→	+	0.57	0.68	0.398	1.691	229	24,992	4.7	Unknown function
eutT	1852	2655	→	+	0.59	0.68	0.298	1.155	267	30,239	6.4	Cobalamin adenosyl transferase
eutD	2652	3668	→	−	0.62	0.68	0.336	1.373	338	36,266	7.5	Resembles substrate domain of Pta
eutM	3709	3999	→	−	0.60	0.67	0.411	2.002	96	9,843	6.5	Carboxysome structural protein?
eutN	4099	4398	→	−	0.57	0.65	0.315	2.094	99	10,351	5.1	Carboxysome structural protein?
eutE	4410	5813	→	−	0.60	0.69	0.354	1.360	467	49,259	6.9	Aldehyde oxidoreductase
eutJ	5824	6663	→	+	0.61	0.72	0.342	1.105	279	30,018	4.9	Possible chaperonin
eutG	6653	7828	→	−	0.57	0.71	0.320	1.429	391	40,570	7.2	Alcohol dehydrogenase
eutH	7948	9195	→	+	0.57	0.73	0.361	1.489	372	39,053	5.8	Membrane protein
eutA	9192	10595	→	−	0.61	0.71	0.297	0.899	467	49,527	5.0	Possible chaperonin
eutB	10607	11968	→	+	0.57	0.73	0.425	1.265	453	49,449	4.7	ET/NH_4[d] subunit
eutC	11987	12883	→	−	0.61	0.70	0.364	1.475	298	32,137	5.9	ET/NH_4 subunit
eutL	12893	13552	→	−	0.60	0.64	0.320	0.841	219	22,696	4.5	Carboxysome structural protein?
eutK	13565	14059	→	−	0.60	0.61	0.289	1.092	164	17,421	7.5	Carboxysome structural protein?
P_R	14078		→									Constitutive internal promoter
eutR	14107	15159	→	−	0.54	0.61	0.280	0.815	350	40,055	7.2	AraC family positive regulator
term	15677		→									Probable terminator
orf79	15828	16619	←	−	0.53	0.54	0.297	0.382	263	30,021	4.8	Unknown
term	16673		←									Probable terminator
orf33	16751	17077	←	−	0.43	0.45	0.201	0.788	109	12,020	8.4	Unknown
hemF	17098	17997	←	−	0.57	0.65	0.353	1.213	299	34,430	6.4	Coproporphyrinogen oxidase
	18000	18869	←		0.51	0.54	0.310	0.555	289	31,659	11.0	*N*-acetylmuramylalanine amidase

[a] Component of the *eut* operon. The vertical line indicates the elements included in the *eut* operon.
[b] The column headed "sense" gives the transcriptional direction, with right-facing arrows indicating counterclockwise on the standard *Salmonella* map; "overlap" indicates whether or not (+ and −, respectively) the stop codon overlaps the initiation codon of the next gene, implying potential translational coupling or interference (40a). Start and stop codon positions are in nucleotides numbered from the end of IS*200*.
[c] GC, fractional G+C content of the gene; 3d.GC, G+C content of codon third positions; CAI, codon adaptation index of Sharp and Li (52a); χ^2, measure of codon usage bias (55a).
[d] ET/NH_4, ethanolamine ammonia lyase.

made by recombination between *eut*::T-POP insertions in the same orientation; these constructed deletions have a T-POP element at the deletion join point which allows induced expression of genes distal to the deletion (see below). Point mutations initially classified by complementation tests and genetic mapping were later sequenced to provide a cross-reference between the genetic and physical maps (Table 5).

Use of T-POP insertions to define gene functions. The transposable element T-POP was derived from transposon Tn*10*dTc (42). Weak tetracycline-inducible transcripts emerge from both ends of the parent transposon Tn*10*dTc (63). Stronger regulated outward transcription is seen for the derived T-POP element because internal transcription terminators have been deleted. When no tetracycline is provided, a T-POP insertion has a strong polar effect on expression of distal genes in an operon, allowing detection of insertions that prevent expression of genes required for a Eut^+ phenotype. Tetracycline induces expression of downstream genes and, in effect, abolishes the polarity effect of the insertion. In the presence of tetracycline, a T-POP insertion is defective only for the gene in which it inserts. Genes with no mutant phenotype can be identified because their T-POP insertions cause a Eut^- phenotype (by a polar effect on distal *eut* genes) that is corrected by addition of tetracycline. This correction is not seen if the target gene is essential to a Eut^+ phenotype.

The *eutS*, *-P*, *-Q*, *-T*, *-D*, *-M*, *-J*, *-G*, *-H*, *-L*, and *-K* genes are not essential for aerobic ethanolamine degradation. Available insertions of T-POP in many genes (*eutSPDMJGJK*) cause a Eut^- phenotype that is corrected by addition of tetracycline (Table 6). In some cases the correction is incomplete, suggesting that the T-POP promoters may not be sufficiently strong to provide a wild-type Eut^+ phenotype; this is frequently true for insertions in orientation B, which directs the weaker *tetR* promoter downstream. Alternatively, the target gene may encode a protein that makes a minor contribution but is not essential to ethanolamine degradation. The phenotypes scored (Table 6) were aerobic use of ethanolamine as a sole carbon and energy source (tested on minimal ethanolamine-vitamin B_{12} plates), and acid production on MacConkey medium containing ethanolamine and vitamin B_{12}.

In-frame spontaneous deletions and constructed deletions with a T-POP insertion at the join point showed phenotypes that helped determine the importance of *eut* genes (Table 7). In-frame deletions should have no polarity effect, and the constructed deletions with T-POP at the junction point have a polarity effect that is corrected by addition of tetracycline. Note that strains lacking the lyase (EutBC) protein show very slight growth on ethanolamine as long as they express the *eutE* gene. This peculiarity reflects a minor secondary route for degradation of ethanolamine that is currently under investigation (see Discussion).

These results confirm the earlier genetic studies showing that the *eutABCE* and *-R* genes are needed for the aerobic Eut^+ phenotype; all other *eut* genes tested have no aerobic

TABLE 2. Key alignments of Eut proteins

Target	Similar protein(s) found	Organism	P(N)[a]
EutS	PduB (organelle structural?)	*Salmonella typhimurium*	0.10
EutP	None		
EutQ	None		
EutT	None		
EutD	Pta (phosphate acetyltransferase)	*Paracoccus denitrificans*	2.0e-85
EutM	PduA (organelle structural?)	*Salmonella typhimurium*	1.1e-37
	CcmK (carboxysome structural)	*Synechocystis* sp. strain PCC6803	7.6e-31
	EutK	*Escherichia coli*	5.3e-22
EutN	CcmL (carboxysome structural)	*Synechococcus*	3.3e-19
EutE	SucD (succinate-semialdehyde dehydrogenase)	*Clostridium kluyveri*	2.0e-36
EutJ	FtsA (cell division)	*Bacillus subtilis*	6.1e-07
	DnaK (heat shock chaperonin)	*Synechocystis* sp. strain PCC6803	2.2e-05
EutG	FucO (lactaldehyde reductase on propanediol oxidoreductase)	*Escherichia coli*	6.7e-66
EutH	YxeR (unknown)	*Bacillus subtilis*	2.2e-105
	MTCY04C12.24c (ABC sulphate transporter?)	*Mycobacterium tuberculosis*	0.064
EutA	FtsA (cell division)	*Bacillus subtilis*	0.25
	DnaK (heat shock chaperonin)	*Thermomicrobium roseum*	0.73
EutB	EutB (ethanolamine ammonia lyase large subunit)	*Rhodococcus erythropolis*	1.5e-140
EutC	EutB (ethanolamine ammonia lyase small subunit)	*Rhodococcus erythropolis*	7.6e-26
EutL	PduB (organelle structural?)	*Salmonella typhimurium*	1.4e-07
EutK	EutM	*Escherichia coli*	4.4e-22
	PduA (organelle structural?)	*Salmonella typhimurium*	4.0e-21
	CcmK (carboxysome structural)	*Synechococcus* sp. strain PCC7942	2.1e-17
EutR	OxoS (putative regulatory protein)	*Pseudomonas putida*	9.4e-18
	ThcR (probable regulatory protein)	*Rhodococcus* NI86/21	5.0e-14
	XylS (transcriptional activator of XylDLEGF operon)	*Pseudomonas putida*	1.9e-12
	TmbS (positive regulator of *tmb*-meta operon)	*Pseudomonas putida*	9.4e-12
	HrpB (positive regulation of hypersensitive response)	*Burkholderia solanacearum*	7.7e-09
	CbdS (positive regulator of *cbd* operon)	*Pseudomonas* sp. strain pBAH1	9.2e-09
	HrpXv (positive regulator of *hrp* cluster)	*Xanthomonas campestris*	1.6e-06
	PocR (positive regulator of *pdu* operon)	*Salmonella typhimurium*	0.084

[a] This score, given by the BLAST program, is the probability that the observed resemblance between entire sequences could occur by chance. For those alignments with high P(N) scores, the relationship is based on shared motifs discussed in the text.

mutant phenotype. No appropriate insertions or deletions were available for testing the phenotype of a *eutN* defect, but since mutations in this gene were not detected in the original genetic analysis, we presume that *eutN*, like the other extra genes, has no aerobic phenotype. The *eutD* gene was initially identified by mutations with a Eut^- phenotype, but multigene deletions that remove the *eutD* gene are phenotypically Eut^+; this suggests that the phenotype of EutD point mutations was due to polar effects on other genes. This will be discussed later.

Homologues of carboxysome proteins. Five genes (*eutS*, *-M*, *-N*, *-L*, and *K*) encode small proteins similar to the shell proteins of the carboxysome, an organelle found in photosynthetic and sulfur-oxidizing bacteria (57). These genes also resemble the *pduA* and *-B* genes of in the *Salmonella pdu* operon, which encode enzymes needed for vitamin B_{12}-dependent degradation of propanediol (10, 11, 18, 47). The *eutM* and *-N* genes were identified previously, and their sequence similarity to carboxysome proteins was noted (60); these genes were originally designated *cchA* and *cchB* and have been renamed, since it is now clear that they are part of the ethanolamine (*eut*) operon.

The EutN protein is very similar to the CcmL protein of *Synechococcus* (Fig. 3). The EutM, EutK, and PduA proteins are clearly homologous to the CcmK protein of *Synechococcus*. The longer EutL, EutS, and PduB proteins are clearly similar to each other and are less obviously related to the others; their distant similarity to the CcmK family (EutMK and PduA) was inferred from a shared multiple-alignment profile (24). The C-terminal portions of the EutL, EutS, and PduB proteins are most similar to that of the PduA protein. Sequence features that the three proteins share with PduA are indicated in Fig. 3; these were identified with the profile program of the Genetics Computer Group sequence analysis software package.

The *eutP* and *eutQ* genes. The predicted EutP and EutQ proteins do not resemble others in current databases. Neither gene has a Eut^- mutant phenotype when mutants are tested aerobically in an otherwise-wild-type background.

The EutP protein has an ATP-GTP binding motif of the P-loop class (Prosite motif PS00017). In addition, a second motif found in EutP was shared with a subset of the proteins containing the first motif. The extended motif is not represented in the Prosite database. Proteins sharing the entire motif either were components of ABC transporters, contributed to antibiotic resistance, or supported bacteriocin pumping, suggesting a transport function.

The *eutT* gene. Point mutations in the *eutT* gene were included in the original set of Eut mutations but appeared to owe their phenotype to polarity on distal genes. This is supported by the fact that all are nonsense mutations (Table 5). This interpretation is consistent with the observation that the most upstream *eutT* point mutations cause a $Eut(N^- C^-)$ phenotype and distal mutations allow the use of ethanolamine as a nitrogen source [$Eut(N^+ C^-)$] (Table 5); this might reflect position-dependent variation in polarity effects. More support for this possibility came from the finding that *rho* polarity suppressors corrected the $Eut(C^-)$ phenotype of *eutT* nonsense mutations (67) and that in-frame *eutT* deletions have no Eut phenotype (see above).

Recently, it was found that lack of EutT function causes a Eut^- phenotype in a *cobA* mutant strain (54) which lacks the

TABLE 3. Positions of *eut* insertion mutations

Locus[a]	Genotype[b]	Strain	Gene position[c]	Operon position[d]
tktB	*zfa-3649*::Tn*10*	TT13441	~1330	~−5640
tnpA	*zfa-3646*::Tn*10* (A)	TT13438	10	−566
	zfa-3645::Tn*10*	TT11568	~340	~−240
tnpA/*eutS*	*zfa-3644*::Tn*10*	TT11567		~60
eutS	*eut-334*::TPOP (A)	TT20356	43	403
eutP	*eut-171*::Mu dJ (B)	TT13752	21	730
	eut-272::TPOP (A)	TT19099	~140	~850
	eut-273::TPOP (B)	TT19100	~140	~850
	eut-274::TPOP (A)	TT19101	~240	~950
	eut-275::TPOP (B)	TT19102	~240	~950
	eut-267::TPOP (A)	TT18814	259	968
	eut-276::TPOP (A)	TT19103	~340	~1050
eutQ	*eut-18*::Mu dA (B)	TT10271	304	1470
eutT	*eut-11*::Mu dA (B)	TT10647	156	2008
	eut-17::Mu dA (B)	TT10653	703	2555
	eut-184::Mu dA (A)	TT13764	733	2585
eutD	*eut-277*::TPOP (B)	TT19104	~200	~2850
	eut-172::Mu dJ (A)	TT13753	409	3061
eutD/*eutM*	*eut-168*::Mu dJ (A)	TT13750		3701
eutM	*eut-278*::TPOP (B)	TT19105	~140	~3850
	eut-271::TPOP (A)	TT19098	~190	~3900
eutN/*eutE*	*eut-6*::Mu dA (B)	TT10642		4407
eutE	*eut-24*::Mu dA (B)	TT10660	~50	~4460
	eut-12::Mu dA (B)	TT10648	~100	~4510
	eut-10::Mu dA (B)	TT10646	~450	~4860
	eut-163::Mu dJ	TT13745	~700	~5110
	eut-279::TPOP (A)	TT19106	~940	~5350
	eut-181::Mu dJ (B)	TT13762	~1020	~5430
eutJ	*eut-269*::TPOP (A)	TT19096	~280	~6100
eutG	*eut-3*::Mu dA (B)	TT10639	~480	~7130
	eut-178::Mu dJ	TT13759	~590	~7240
	eut-4::Mu dA (B)	TT10640	~780	~7430
	eut-26::Mu dA (B)	TT10662	~960	~7610
	eut-270::TPOP (A)	TT19097	976	7628
	eut-173::Mu dJ (B)	TT13754	~1040	~7690
	eut-20::Mu dA (B)	TT10656	~1080	~7730
eutG/*eutH*	*eut-160*::Mu dJ (B)	TT13742		~7890
	eut-154::Mu dJ (B)	TT13737		~7890
eutH	*eut-9*::Mu dA (B)	TT10645	~80	~8030
	eut-203::Mu dJ (B)	TT13778	~140	~8090
	eut-25::Mu dA (B)	TT10661	508	8456
	eut-21::Mu dA (B)	TT10657	~680	~8630
	eut-22::Mu dA (B)	TT10658	~680	~8630
	eut-192::Mu dJ (B)	TT13769	~1130	~9080
eutA	*eut-208*::Tn*10*dTc (B)	TT10644	~5	~9200
	eut-176::Mu dJ (A)	TT13757	~350	~9540
	eut-1::Mu dA (B)	TT10637	~690	~9880
	eut-183::Mu dJ (B)	TT13763	~690	~9880
	eut-280::TPOP (A)	TT19107	~960	~10150
eutB	*eut-5*::Mu dA (B)	TT10641	~10	~10610
	eut-8::Mu dA (B)	TT10644	~710	~11310
	eut-281::TPOP (A)	TT19108	~940	~11550
	eut-282::TPOP (A)	TT19109	~1040	~11650
eutC	*eut-2*::Mu dA (B)	TT10638	48	12035
	eut-283::TPOP (B)	TT19110	~460	~12450
eutL	*eut-284*::TPOP (A)	TT19111	~60	~12950
	eut-15::Mu dA (B)	TT10651	64	12957
	eut-23::Mu dA (B)	TT10659	73	12966

Continued on following page

TABLE 3—*Continued*

Locus[a]	Genotype[b]	Strain	Gene position[c]	Operon position[d]
	eut-177::Mu dA (B)	TT13758	108	13001
	eut-34::Mu dA (B)	TT10670	117	13010
eutK	*eut-268*::TPOP (A)	TT18828	~340	~13900
	eut-285::TPOP (B)	TT19112	~340	~13900
	eut-286::TPOP (A)	TT19113	~390	~13950
eutR	*eut-156*::Mu dJ (B)	TT13738	28	14135
	eut-205::Tn*10*	TT13893	862	14969
eut trailer	*eut-38*::Mu dA (B)	TT10674		15364
Outside *eut* operon	*zfa-3648*::Tn*10*	TT13440		15930

[a] Insertions with two gene designations (X/Y) are in the interval between the indicated genes.
[b] "(B)" after a Mu-derived element indicates that the *eut* operon was fused to the *lac* operon of the element; "(A)" indicates the opposite orientation. An "(A)" after a Tn*10*-derived element indicates that tetracycline-regulated expression of downstream *eut* genes is from the *tetA* promoter of the T-POP element; "(B)" implies the opposite orientation. For both Mu- and Tn*10*-derived elements, no symbol means that the orientation was not determined.
[c] Nucleotide position within the affected gene. ~, positions were determined by sizing PCR fragments and are approximate; other positions were exactly determined by sequencing.
[d] Distances are from the proximal end of the IS*200* insertion at the left end of the operon (Fig. 1).

general cobalamin adenosyl transferase (26, 61, 62). The *eutT* gene appears to encode a second cobalamin adenosyl transferase, which converts CNB_{12} to $AdoB_{12}$ (the lyase cofactor) (54). The *pdu* operon of *Salmonella* also appears to encode an adenosyl transferase (*pduG*) (1, 9, 66) which is very similar in sequence to a demonstrated adenosyl transferase (OrfZ) in the diol dehydratase operon of *Citrobacter* (22, 52). Surprisingly, the amino acid sequence of the EutT protein shows no similarity to that of the CobA adenosyl transferase (20, 23, 61) or to that of the adenosyl transferase PduG/OrfZ associated with diol dehydratase operons in *Salmonella* and *Citrobacter* (9, 52). Thus, it appears that three extremely different enzymes are able to catalyze adenosylation of cobalamin—EutT, CobA, and PduG/OrfZ.

The *eutD* gene. Some point mutations in the *eutD* gene have a Eut(N^- C^-) phenotype, and others are Eut(N^+C^-) (44, 45). Point mutations in this gene constituted a clear complementation group in the original genetic tests; they complemented mutants in all other genes and did not cause a measurable decrease in the level of ethanolamine ammonia lyase or acetaldehyde dehydrogenase, encoded by distal genes (45). These point mutations were assigned to an open reading frame by correlating map positions with the physical locations of insertion sites determined by PCR; their location was confirmed by

TABLE 4. Deletions

Deletion	Nucleotides[a]	Gene(s)	Strain	Source
Del913	(−)566 . . .	All of *eut-cysA*	TT14526	Tn*10* × Tn*10*
Del1955	(−)566–15930	All of *eut*	TT20606	Tn*10* × Tn*10*
Del763	730 . . .	*eutS171–cysA*	TT11734	Mu dA × Mu dA
Del744	1470 . . .	*eutQ18–cysA*	TT11715	Mu dA × Mu dA
Del739	2008 . . .	*eutT11–cysA*	TT11710	Mu dA × mu dA
Del743	2555 . . .	*eutT17–cysA*	TT11714	Mu dA × Mu dA
Del762	2585 . . .	*eutT184–cysA*	TT11733	Mu dA × Mu dA
Del734	4407 . . .	*eut6–cysA*	TT11705	Mu dA × Mu dA
Del752	8456 . . .	*eutH25–cysA*	TT11723	Mu dA × Mu dA
Del730	12035 . . .	*eutC2–cysA*	TT11701	Mu dA × Mu dA
Del741	12957 . . .	*eutL15–cysA*	TT11712	Mu dA × Mu dA
Del750	12966 . . .	eutL23–cysA	TT11721	Mu dA × Mu dA
Del756	13001 . . .	*eutL177–cysA*	TT11727	Mu dA × Mu dA
Del747	13010 . . .	*eutL34–cysA*	TT11718	Mu dA × Mu dA
Del754	14135 . . .	*eutR156–cysA*	TT11725	Mu dA × Mu dA
Del863	15364 . . .	*eut-38–cysA*	TT13783	Mu dA × Mu dA
eut-333	750–2919	*eutP–eutD*	TT20581	In-frame deletion
eut-302	2692–3994	*eutD–eutM*	TT19189	In-frame deletion
eut-300	6646–7438	*eutJ–eutG*	TT19187	In-frame deletion
eut-301	6945–8362	*eutG–eutH*	TT19168	In-frame deletion
eut-339	403–~850	*eutS–eutP*	TT20586	T-POP × T-POP
eut-340	403–~5350	*eutS–eutE*	TT20587	T-POP × T-POP
eut-336	403–~13950	*eutS–eutK*	TT20583	T-POP × T-POP
eut-338	~5350–~13950	*eutE–eutK*	TT20585	T-POP × T-POP
eut-337	~6100–~13950	*eutJ–eutK*	TT20584	T-POP × T-POP
eut-335	~10150–~13950	*eutA–eutK*	TT20582	T-POP × T-POP

[a] All positions are relative to the base immediately distal to the IS*200* insertion (Fig. 2); numbers preceded with (−) are to the left of that reference point. ~, numbers are approximate positions determined by the sizes of PCR fragments or fine-structure mapping.

TABLE 5. Point mutations

Allele	Mutation		Strain	Phenotype
	Amino acid[a]	Codon		
eutT77	Q61Am	CAG→TAG	TT11494	*eut*(N)$^-$
eutT62[b]	Q62Am	CAG→TAG	TT11479	*eut*(N)$^-$
eutT86	P76L,Q77Am	CCACAG→CTATA	TT11503	*eut*(N)$^-$
eutT67	Q77Am	CAG→TAG	TT11484	*eut*(N)$^+$
eutT10	W125Op	TGG→TGA	TT11518	*eut*(N)$^+$
eutT75	Q127Oc	CAA→TAA	TT11492	*eut*(N)$^+$
eutT78	Q134Op	TGG→TGA	TT11406	*eut*(N)$^+$
eutT74	Q177Am	CAG→TAG	TT11491	*eut*(N)$^+$
eutD64	Q18Am	CAG→TAG	TT11481	*eut*(N)$^+$
eutD53	Q35Oc	CAA→TAA	TT11470	*eut*(N)$^-$
eutD10	Q156Am	CAG→TAG	TT11515	*eut*(N)$^+$
eutD12	R173Op	CGA→TGA	TT11538	*eut*(N)$^+$

[a] Original amino acid (left) and substitution (right) flank the position of the affected codon in the gene. Nonsense codons are abbreviated as follows: Am, amber (TAG); Op, opal (TGA); Oc, ocher (TAA).
[b] Unlisted mutations *eutT88* (TT11505) and *eutT90* (TT11507) are recurrences of the same mutation as listed mutation *eutT62*, i.e., Q184Am.

sequencing (Table 5). However the finding that all of the *eutD* point mutations are nonsense types made it reasonable that their phenotype might have been due to polarity effects.

A *eutD*::T-POP insertion mutant remains phenotypically Eut(C$^-$) (on minimal medium) even when tetracycline is added to induce downstream genes, but tetracycline restores the ability to produce acid, suggesting partial correction (Table 6). Unfortunately, the only available *eutD*::T-POP insertion is in the B orientation, which provides only weak induction of distal functions. These results make it difficult to decide whether the phenotypes seen for *eutD* mutations are due to polarity effects or an inherent lack of EutD function. However, the nonpolar deletion mutations (*eutPQTD* and *eutDM*), which remove both the *eutD* gene and additional adjacent material, are Eut$^+$ aerobically. The simplest interpretation is that *eutD* point mutations owe their phenotype to polarity effects on multiple downstream genes and a simple EutD defect causes no aerobic phenotype.

TABLE 6. Phenotypes of *eut*::T-POP insertions

Gene	Allele[a]	Operon location[b]	Strain no.	Growth on NCE ethanolamine B_{12}[c]		Color on MacConkey ethanolamine B_{12}[d]	
				−Tc	+Tc	−Tc	+Tc
eutS	*eut-334* (A)	403	TT20357	0	3	0	4
eutP	*eut-272* (A)	~850	TT19099	0	3	0	4
	eut-273 (B)	~850	TT19100	0	0	0	0
	eut-274 (A)	~950	TT19101	0	0	0	3
	eut-275 (B)	~950	TT19102	0	1	0	0
	eut-267 (A)	968	TT18814	0	1	0	4
	eut-276 (A)	~1050	TT19103	0	1	0	4
eutD	*eut-277* (B)	~2850	TT19104	0	0	0	2
eutM	*eut-278* (B)	~3850	TT19105	0	0	0	3
	eut-271 (A)	~3900	TT19098	0	0	0	4
eutE	*eut-279* (A)	~5350	TT19106	0	0	0	0
eutJ	*eut-269* (A)	~6100	TT19096	0	3	0	3
eutG	*eut-270* (A)	7628	TT19097	0	3	0	3
eutA	*eut-280* (A)	~10150	TT19107	0[e]	0[e]	0	1[e]
eutB	*eut-281* (A)	~11550	TT19108	0[f]	0[f]	0	0[f]
	eut-282 (A)	~11650	TT19109	0[f]	0[f]	0	0[f]
eutC	*eut-283* (B)	~12450	TT19110	0[f]	0[f]	0	0[f]
eutL	*eut-284* (A)	~12950	TT19111	1	3	2	3
eutK	eut-268 (A)	~13900	TT18828	0[g]	3	3	4
	eut-285 (B)	~13900	TT19112	0[g]	3	3	4
	eut-286 (A)	~13950	TT19113	0[g]	3	3	4

[a] A, induced transcription is from the *tetA* promoter of the T-POP element; B, T-POP insertion is in the opposite orientation.
[b] Nucleotide position is distance from the first nucleotide at the left of IS*200*V. ~, approximate location determined by PCR; other positions are by direct sequence determination.
[c] Growth is scored on solid minimal medium with ethanolamine as sole carbon source. 0, no growth; 1, slow growth; 2, moderate growth; 3, growth as strong as wild type.
[d] The Eut phenotype can be scored by acid production, which produces a red color on this medium. 0, white; 1, pale pink; 2, pink; 3, red; 4, dark red.
[e] In *eutA* mutants, the EutBC lyase is inhibited by CNB_{12} at the concentration used here; thus, *eutA* mutants show weak growth similar to that of *eutBC* mutants.
[f] Strains that lack the *eutBC* (lyase) but still express the *eutE* gene are scored here as 0; they show extremely weak but reliably scorable growth that is due to a minor (EutE-dependent) secondary pathway of ethanolamine degradation.
[g] The "0" response of eutK insertions without tetracycline may reflect blockage of *eutR* transcription from a weak internal promoter between the eutL and eutK insertions used here.

TABLE 7. Aerobic phenotypes of *eut* deletions

Class[a]	Deletion	Removed	Strain	Growth[b]	
				−Tc	+Tc
1	*eut*$^+$ (Tc^r)		TT13440	3	
	Del1955	All *eut*	TT20606	0	
	Δ*eut-300*	*eutJ–eutG*	TT19187	3	
	Δ*eut-301*	*eutG–eutH*	TT19188	3	
	Δ*eut-302*	*eutD–eutM*	TT19189	3	
	Δ*eut0333*	*eutP–eutD*	TT20582	3	
2	*eut*$^+$ (Tc^r)		TT13440	3	3
	Δ*eut-338 (T-POP)*	Δ*eutE–eutK*	TT20585	0[c]	0
	Δ*eut–335* (T-POP)	Δ*eutA–eutK*	TT20582	0	0
	Δ*eut-336* (T-POP)	*eutS–eutK*	TT20583	0	0
	Δ*eut-337* (T-POP)	*eutJ–eutK*	TT20584	0	0
	Δ*eut-339* (T-POP)	*eutS–eutP*	TT20586	0	2
	Δ*eut-340* (T-POP)	*eutS–eutE*	TT20587	0	0
	Δ*eut-302*, Δ*eut-337* (T-POP)	*eutD–eutM*, *eutJ–eutK*	TT20589	0	0
	Δ*eut-333* Δ*eut-337* (T-POP)	*eutP–eutD*, *eutJ–eutK*	TT20588	0	0

[a] Class 1 deletions are in-frame deletions derived from T-POP insertions. They retain no Tn*10* material and show no polar effect on downstream *eut* genes. Class 2 deletions were constructed by recombination between T-POP insertions in different *eut* genes. They carry a T-POP insertion at the deletion junction point and are strongly polar but express distal *eut* genes in the presence of tetracycline.

[b] Growth responses are scored as follows: 0, no growth; 1, very weak; 2, intermediate; 3, like wild type. Strains that express EutE but not EutBC (lyase) are listed as 0; they show extremely weak (EutE-dependent) growth that is due to a secondary minor route for conversion of ethanolamine to acetaldehyde (see Discussion). Tc, tetracycline.

[c] Strains that lack the *eutBC* (lyase) gene but still express the *eutE* gene are scored here as 0; they show extremely weak but reliably scorable growth that is due to a minor (EutE-dependent) secondary pathway of ethanolamine degradation.

The predicted EutD protein sequence is very similar to the C-terminal half of Pta (phosphotransacetylase) and MeaB (NADP-dependent malate oxidoreductase, or malic enzyme). The Pta enzyme catalyzes conversion of acetyl-CoA to acetyl phosphate, and MeaB catalyzes the conversion of malate to pyruvate with release of CO_2.

The function of the domain shared by these three proteins is not known, but we suspect that it may provide substrate specificity rather than catalytic activity. Several malate oxidoreductases align only with the N-terminal domains of Mez and Pta and share no similarity with EutD protein (e.g., *Streptococcus bovis* [GenBank accession no. U35659]); the substrate specificities of these single-domain proteins are reportedly relaxed. Similarly, several malate-decarboxylating enzymes, malic enzymes (which produce pyruvate), and malolactic enzymes (which produce lactate) resemble the N-terminal domain of Pta but lack the C-terminal domain that is homologous to the EutD sequence. Because the two classes of homologues of Pta and Mez enzymes seem to have catalytic domains which are not similar to EutD, we suspect that EutD is not an independent catalyst but may serve as a subunit of a larger complex, perhaps one involved in CO_2 fixation.

The *eutE* and *eutG* genes (an aldehyde dehydrogenase and an alcohol dehydrogenase). The *eutE* gene was initially identified in mutants which could use ethanolamine as a source of nitrogen but not carbon (45). Direct assay revealed that these mutants lack acetaldehyde dehydrogenase, which converts acetaldehyde to acetyl-CoA (44). The gene was initially sequenced by Stojiljkovic et al. (60), who noted that the predicted amino acid sequence of the protein was strikingly similar to that of the aldehyde oxidoreductase domain of the AdhE family of alcohol dehydrogenases-aldehyde oxidoreductases. Sequencing of mutations in the *eutE* complementation group demonstrated that they affect this open reading frame. The EutE sequence most closely resembled that of NADP-dependent succinate-semialdehyde dehydrogenase of *Clostridium kluyveri*, which catalyzes formation of succinyl-CoA (59).

The EutG protein appears to be an alcohol dehydrogenase (aldehyde reductase) (60). The best BLAST alignment was with lactaldehyde reductase (1,2-propanediol oxidoreductase) of *E. coli*. The EutE and EutG sequences aligned in tandem without overlap along the *E. coli* AdhE sequence, with EutE resembling the C-terminal aldehyde oxidoreductase domain and EutG resembling the N-terminal alcohol dehydrogenase domain. The AdhE protein is known to catalyze reduction of acetyl-CoA to acetaldehyde and further to ethanol. We propose that the EutE and EutG proteins together catalyze the same reactions as AdhE. During growth on ethanolamine, EutE catalyzes formation of acetyl-CoA (as shown previously) and EutG may help to maintain redox balance by reducing some aldehyde to ethanol. Mutants of the *eutG* gene have no Eut phenotype under the conditions tested, presumably because $NADH^+$ can be recycled via respiratory enzymes or other alcohol dehydrogenases (Fig. 1). In the *eut* operon, the tandem arrangement of the *eutE* and *eutG* genes is interrupted by the *eutJ* gene.

The *eutJ* and *eutA* genes may encode chaperonins. The *eutJ* gene had no mutant phenotype. The inferred amino acid sequence of the EutJ protein showed similarity to that of members of the DnaK family of heat shock chaperonins (60). A comparison of the conserved cores of EutJ, EutA, and the *E. coli* DnaK protein is shown in Fig. 4.

Mutations in the *eutA* gene cause a distinct Eut(N^- C^-) phenotype under aerobic conditions with CNB_{12} and defined one of the original *eut* complementation groups (45). These mutants became phenotypically Eut(N^+ C^-) when $AdoB_{12}$ was provided instead of CNB_{12}. A *eutA* mutant shows normal induction of the operon by CNB_{12} or $AdoB_{12}$, demonstrating that it is not defective for cobalamin adenosylation (54). Recent results suggest that EutA protects the lyase from inhibition by CNB_{12} (54). It is important to remember that *eutA* mutants retain their Eut(C^-) phenotype even when $AdoB_{12}$ is provided, suggesting that the protein plays some additional role.

The EutA sequence is weakly related to the same group of proteins that show similarity with EutJ (Table 2 and Fig. 4). A

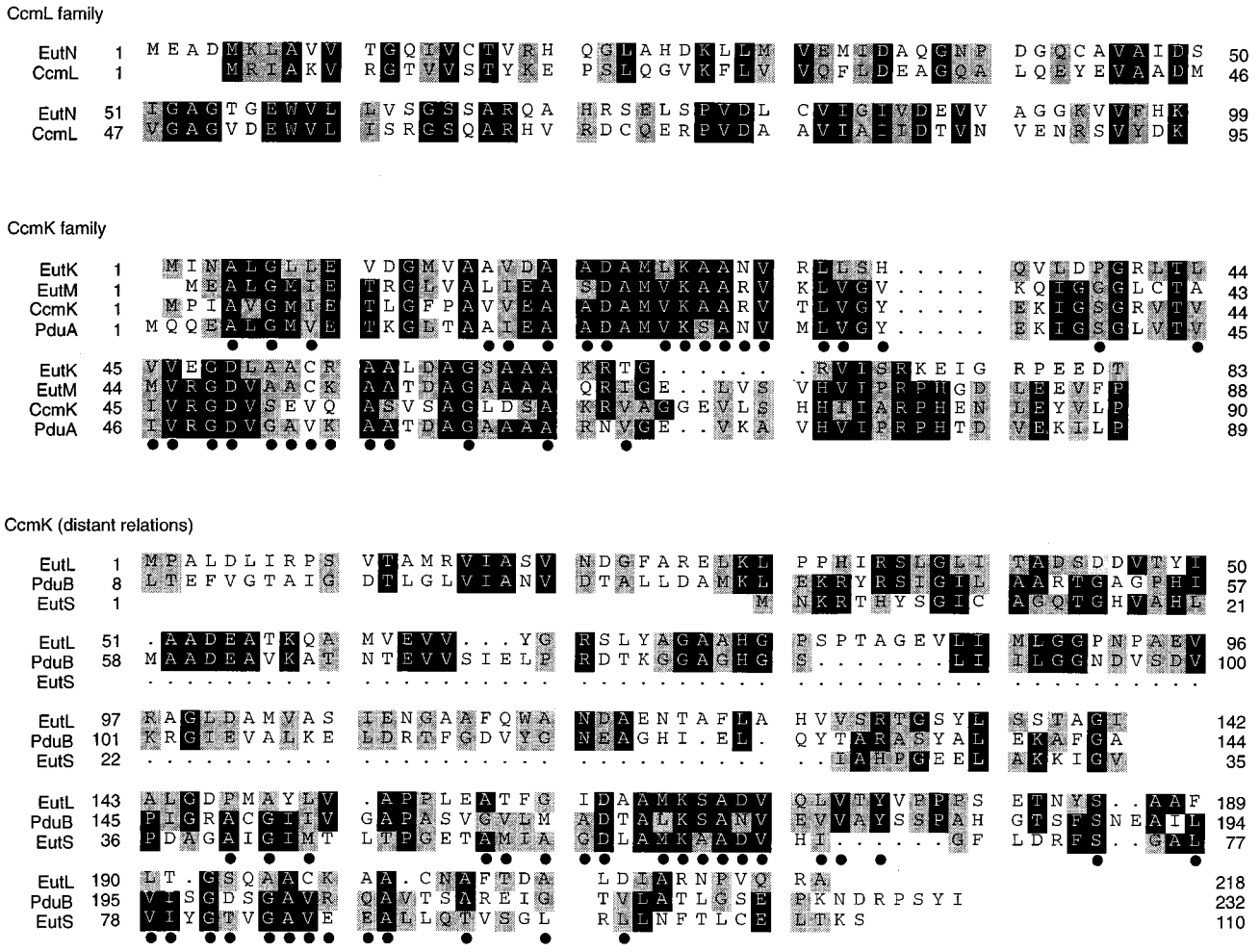

FIG. 3. Alignment of carboxysome shell protein homologues. The top panel shows the alignment of the EutN protein with the CcmL protein of *Synechococcus*. The middle panel aligns the EutK and EutM proteins with the CcmK protein of *Synechococcus* and the PduA protein of the *Salmonella pdu* (propanediol utilization) operon. The bottom panel aligns the EutL and EutS proteins with the PduB protein from the *Salmonella pdu* operon. The EutLS family is most similar to the PduA protein of the EutK, EutN, CcmK class. The sequence features shared by the EutLS-PduB family and the PduA protein are indicated by the black dots below the sequences.

motif common to EutJ, EutA, and the DnaK family proteins was the tract DIGGGT. This sequence pattern is part of the nucleotide binding loop in the crystal structure of DnaK protein (55).

The EutJ and EutA proteins may be important in assembling the carboxysome or in refolding lyase. The adenosyl moiety of $AdoB_{12}$ is cleaved during catalysis (2) and may be subject to occasional loss from the enzyme or destruction by inappropriate reactions (65). Cobalamins without adenosine bind strongly to the enzyme and inhibit its activity in vitro (7). Replacement of damaged $AdoB_{12}$ may require removal by refolding lyase. The ability to remove inhibitory forms of vitamin B_{12} from lyase may contribute to the ability of EutA to protect lyase from inhibition. A function of this sort has been reported for the vitamin B12-dependent enzyme propanediol dehydratase (65).

The *eutH* gene encodes a membrane protein of unknown function. The *eutH* gene had no mutant phenotype. The deduced EutH amino acid sequence suggests 11 membrane-spanning segments capped at their ends with short tracts of polar

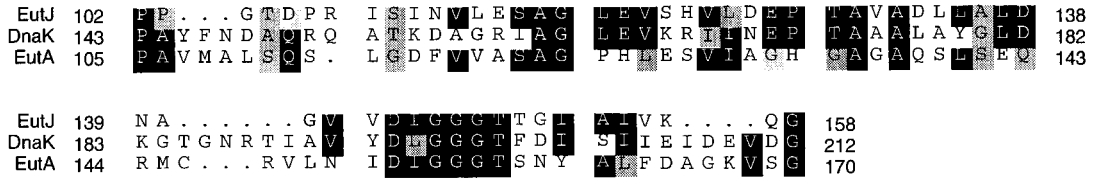

FIG. 4. Sequence motif that EutJ and EutA proteins share with the chaperonin DnaK. Only EutJ shows significant similarity to DnaK over its entire length; EutJ and EutA are not significantly similar to each other, but they share the motif mentioned above. The central DIGGT motif is part of a nucleotide binding loop in the DnaK protein (55).

residues. Although a role in ethanolamine transport has been suggested for the EutH protein (60), genetic data indicate that no ethanolamine transport functions are encoded within the operon (45). However, if sufficient ethanolamine enters cells by other means, this gene could encode a transporter with a very slight mutant phenotype. This is true for the propanediol diffusion facilitator PduF, which makes only a minimal contribution to the ability of cells to grow on that carbon source (18, 19). Unlinked mutations previously thought to affect ethanolamine transport have recently been shown to affect vitamin B_{12} uptake (41, 64). The EutH protein has no resemblance to a reported ethanolamine transporter, EutP, from *Rhodococcus* (GenBank accession no. U17129). Other possibilities are that the EutH protein increases uptake of vitamin B_{12} or facilitates efflux of acetaldehyde or acetate produced during ethanolamine catabolism.

The *eutBC* genes encode ethanolamine ammonia lyase. The assignment of ethanolamine ammonia lyase to the *eutBC* genes was initially based on enzyme assays of mutants for these two genes (44). The cloned sequence that complemented these two mutant types provided the first sequence for an ethanolamine ammonia lyase (27). The sequence data reported here contain several corrections of the originally reported sequence. Use of the improved sequence may help identify cobalamin binding motifs (38). The only other described homologue of lyase is from *Rhodococcus* (GenBank accession no. L24492), whose *eutB* and *eutC* homologues are adjacent but do not appear to be part of a larger operon.

The EutR protein is a positive regulatory protein of the AraC family. The *eutR* gene was identified in mutants with a Eut^- phenotype that were unable to induce the operon in response to the regulatory effectors, ethanolamine and vitamin B_{12} (45, 46). The EutR protein is encoded within the operon and thus positively controls its own synthesis. This autocatalytic cycle is essential for full operon induction. Coinduction of lyase (EutBC) and EutR may serve to equalize their competition for a small pool of $AdoB_{12}$, allowing operon control to remain sensitive to cofactor levels over a wide range of vitamin B_{12} concentrations (53).

The EutR protein is similar in amino acid sequence to a variety of known regulatory proteins in the AraC family. As is typical for this family, the similarity is restricted to the C-terminal helix-turn-helix domain. Since operon transcription is induced only in the presence of both ethanolamine and vitamin B_{12}, the EutR protein may bind both effectors. This has not been demonstrated experimentally and places heavy demands on the EutR protein to recognize two effectors, a DNA binding site and components of the transcription apparatus. It would simplify matters if the requirement for vitamin B_{12} induction were to help convert ethanolamine to acetaldehyde, which served as sole inducer. However, mutants that lack lyase show normal operon induction by vitamin B_{12} plus ethanolamine, consistent with direct recognition of the two effectors (46).

The region between the *eut* operon and the *hemF* gene. The region between the *eut* operon and the *hemF* gene includes a sequence resembling a Rho-independent transcription terminator located 519 bases from the end of the *eutR* gene (Fig. 2 and Table 1). A heavily exploited Mud-*lac* insertion mutant (*eut-38*::MudA) lies between the last gene in the operon (*eutR*) and this proposed terminator (Table 3). Strains with this insertion are phenotypically Eut^+ but show β-galactosidase induction in response to *eut* operon regulatory effectors (46). This insertion lies within the transcribed region of the operon but promoter distal to all structural genes.

In *Salmonella*, two open reading frames (Orf79 and Orf33) are found between the *eut* operon and the nearby *hemF* gene. The orientation of the *hemF* gene, Orf33, and Orf79 is opposite to that of the *eut* operon. In *E. coli*, only 5 nucleotides separate the *eutR* and *hemF* coding sequences (Fig. 2); each transcript appears to be terminated by a rho-dependent terminator located within the coding sequence of the neighboring gene. A potential transcription terminator for Orf33 was found between Orf33 and Orf79. No significant alignments were found between the translated product of Orf79 or Orf33 and proteins in the database.

The region upstream of the *eut* operon. The 1,200 bases upstream of the first gene of the *eut* operon (*eutS*) includes one of the six IS*200* elements found in the chromosome of *S. typhimurium* LT2 (32, 35, 48). The element is flanked by pairs of A residues, as seen in other examples of IS*200* insertions (31). Upstream of the insertion sequence is the *meaB* gene, encoding NADP-dependent malic enzyme (malate → pyruvate) (39, 40). The *meaB* gene and the IS*200* element are separated by 42 bases. To the left of *meaB* are the genes (*tktB* and *talA*) for transketolase and transaldolase, enzymes which act in the pentose-phosphate shunt. They form an apparent operon whose orientation is opposite to that of the *eut* and *meaB* genes.

The *eut* operon has a main promoter and a minor internal promoter. The main regulated promoter (P_I) is activated by EutR when both ethanolamine and $AdoB_{12}$ are present and requires Crp protein as a global regulator (46). A good potential σ^{70} binding site was found 83 nucleotides before the start of the *eutS* gene. This lies within a noncoding region well conserved between *Salmonella* and *E. coli*. We assume, but have not yet demonstrated, that the EutR regulator binds a site within this region to stimulate transcription. Although the operon is subject to catabolite repression (46), we have found no likely Crp-binding site in the sequence in this region.

The second promoter (P_{II}) lies adjacent to the *eutR* gene and appears to provide a low constitutive level of EutR regulator sufficient to initiate induction of the main promoter (46, 53). Location of the P_{II} promoter in the *Salmonella* operon was determined by mRNA runoff extension primed by oligonucleotides complementary to mRNA sequence within the *eutR* gene (data not shown). This message starts 29 bases before the beginning of *eutR* in the *eutKR* interval. The existence of this promoter does not preclude the existence of additional weak promoters further upstream which might contribute to the basal level of EutR protein. No obvious σ^{70} consensus is associated with P_{II}.

Comparing the *eut* operons of *S. typhimurium* and *E. coli*. Initial biochemical work on ethanolamine degradation was done for *E. coli*, with little parallel genetic analysis. Both *S. typhimurium* and *E. coli* use the same degradative pathway, and both sets of enzymes are induced by the presence of ethanolamine plus vitamin B_{12} (6, 7, 33, 34). As diagrammed in Fig. 2, the *E. coli* operon sequence encodes close homologues of the 17 genes described above for *Salmonella* (8). The presence of a *eut* operon in *E. coli* is surprising in that *E. coli* does not make the needed vitamin B_{12} cofactor de novo (36, 37). Furthermore, *E. coli* cannot reduce tetrathionate, a process that seems essential for anaerobic ethanolamine degradation by *Salmonella*. For both organisms, the *eut* operon has a rather high G+C content, suggesting acquisition by horizontal transfer. However, the two sequences differ at only 17% of aligned positions, a degree of conservation expected for genes that have been inherited vertically from the common ancestor of *Salmonella* and *E. coli*. A surprising feature of the *E. coli eut* operon sequenced by Blattner and coworkers (8) is the presence of an insertion element between the *eutA* and *eutB* genes

which does not damage either of the flanking genes (Fig. 2). This element is not found in other K-12 genomes (69).

DISCUSSION

The complete sequence of the *eut* operon includes 17 genes, of which only 6 are required for aerobic use of ethanolamine as a carbon or nitrogen source. The functions encoded by the extra genes may be needed for ethanolamine use under unknown conditions, or they may make a slight contribution that escaped our detection. We initially expected that the extra genes would be required for anaerobic growth. This has recently been tested, since it was found that wild-type strains can grow anaerobically on ethanolamine if the electron acceptor tetrathionate is provided (12). However, the extra *eut* genes tested thus far are also nonessential for anaerobic growth with tetrathionate (28). It seems that the lack of mutant phenotypes for the extra genes is due to an alternative pathway for ethanolamine degradation that can supply some of the Eut functions and prevent the detection of *eut* mutations in some genes. In the presence of mutations that appear to block this alternative pathway, all genes in the *eut* operon have a Eut^- phenotype aerobically and anaerobically (28).

The five Eut proteins that are similar to carboxysome components suggest that the ethanolamine pathway may involve fixation of CO_2. In photosynthetic bacteria (*Synechococcus*) and in sulfur oxidizers (*Thiobacillus*), this protein-bounded organelle is thought to concentrate CO_2 and exclude O_2; this supports activity of RUBISCO, the enzyme directly involved in CO_2 fixation (14, 25, 29, 30, 56, 58). In *Salmonella*, structures resembling carboxysomes have recently been observed by electron microscope following induction of the *pdu* (9) or *eut* (21) operon, but fixation of CO_2 has not yet been shown to accompany growth on ethanolamine.

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